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Folate is an essential dietary vitamin involved in methylation reactions and DNA synthesis. Insufficient dietary folate has been associated with various developmental and adult diseases such as neural tube defects and colorectal cancer. The basis for these associations has not been clearly defined. In microarray studies, components of the Wnt signaling pathway have been shown to be altered under folate-deficient conditions. The Wnt signaling pathway has multiple functions, affecting cell growth, differentiation, and development. The purpose of this study was to determine if folate deficiency leads to altered Wnt signaling with a focus on β-catenin activity, protein levels, and cellular localization. NIH3T3 cells containing the TOPFlash β-catenin luciferase reporter were grown for 4, 8, and 10 days in folate-deficient and sufficient custom DMEM medium either with or without activation of the Wnt pathway by addition of Wnt3a conditioned medium. β-catenin activity, as measured by luciferase activity per unit DNA, was higher in folate-deficient cells at 8 and 10 days in Wnt3a stimulated cells. β-catenin protein levels as assayed by Western blot analysis were not significantly different in folate-deficient cells. Indirect immunofluorescence revealed increased nuclear localization of β-catenin in folate-deficient cells of both Wnt3a stimulated and unstimulated cells at 10 days. Overall, these results suggest that folate deficiency alters cell function, in part, due to changes in Wnt signaling.

THE EFFECT OF FOLATE DEFICIENCY ON

THE WNT SIGNALING PATHWAY

By

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Master of Science

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Approved by

_____
Committee Chair

To my parents, Yves and Fay Morillon, without whose support and encouragement, I would not have succeeded in my endeavors.

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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Date of Acceptance by Committee

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Date of Final Oral Examination

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I would like to thank my advisor and committee chair, Dr. Karen Katula for supporting me in my thesis research and providing me with direction and knowledge, without which this work would not have been possible. I would like to also thank the members of my thesis committee, Dr. John Tomkiel and Dr. Yashomati Patel for their suggestions and dedication to making this a successful project.

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CHAPTER I

INTRODUCTION

Statement of Problem

The purpose of this study is to determine if folate deficiency leads to alterations in the Wnt signaling pathway.

Folate Uptake and Metabolism

Folate is a necessary vitamin that must be obtained from the diet. It is found in leafy green vegetables, citrus fruits, and organ meats. Folate can also be obtained through dietary supplementation in the form of folic acid. Once ingested, folate is taken up by enterocytes of the small intestine and enters the blood. Plasma folate is bound to various proteins for transport and its level is regulated by the liver. As folate passes through the blood it binds to the specific cell surface transporters Folate Binding Protein (FBP) and Folate co-Transporter (FcoT) and is transported into the cell.

Intracellular folate is converted into 7,8 dihydrofolate, which is then converted into tetrahydrofolate via dihydrofolate reductase (Fig. 1a). Tetrahydrofolate is converted into 5,10-methylenetetrahydrofolate, which can then be converted into 5-methyl-tetrahydrofolate via 5,10-methylenetetrahydrofolate reductase. The metabolite, 5,10-methylene tetrahydrofolate, is used to convert deoxyuridylate monophosphate into thymidylate monophosphate, a required nucleotide used in DNA synthesis (Fig. 1b). 5-

methyl-tetrahydrofolate is required for the conversion of homocysteine into methionine, which in turn is converted into S-adenosylmethionine, the major methyl donor in methylation reactions of DNA, proteins, and lipids (Ulrich, 2005).

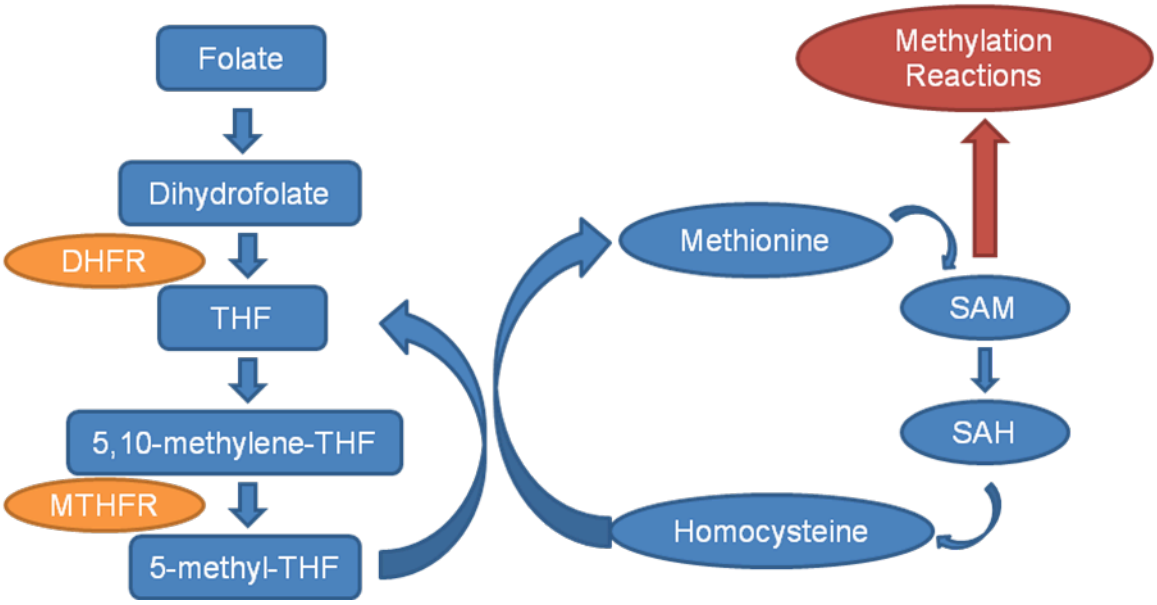


Fig. 1. a) Methylation of Homocysteine to Methionine. Dihydrofolate reductase (DHFR), methylenetetrahydrofolate reductase (MTHFR), Tetrahydrofolate (THF), S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH) (Modified from Ulrich, 2005)

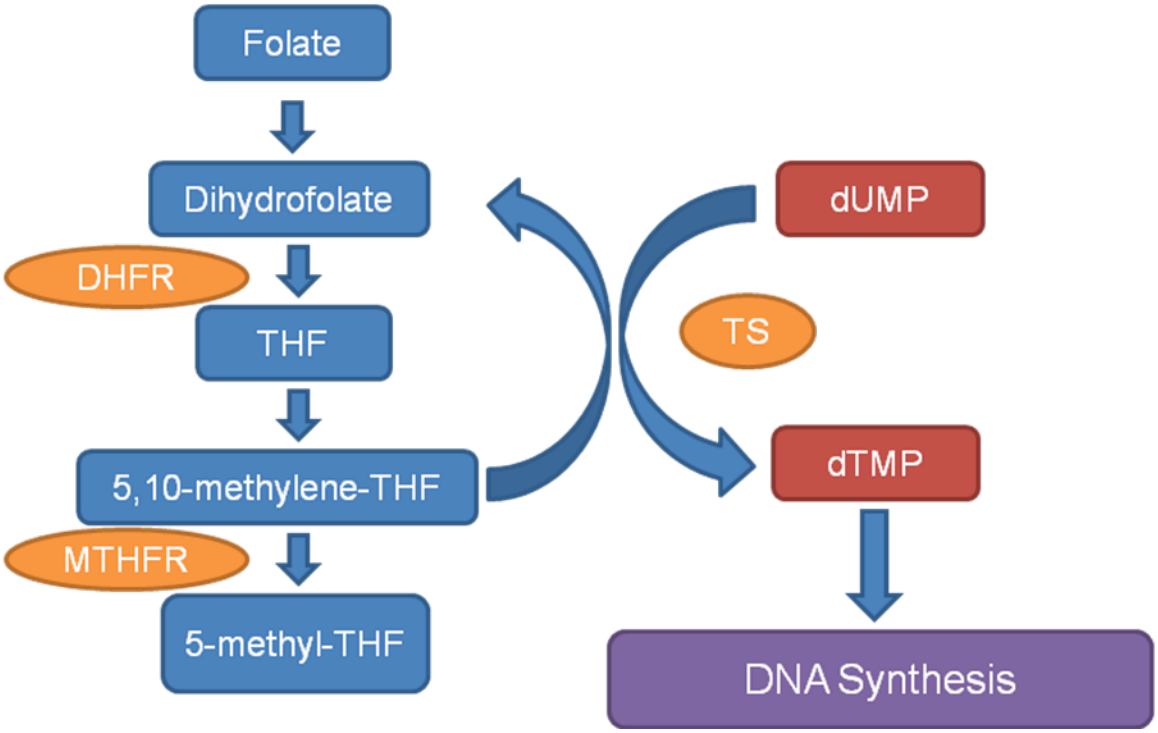


Fig. 1. b) Purine and Pyrimidine Biosynthesis. Dihydrofolate reductase (DHFR), methylenetetrahydrofolate reductase (MTHFR), Tetrahydrofolate (THF), Deoxyuridylate monophosphate (dUMP), thymidylate monophosphate (dTMP), Thymidylate synthase (TS) (Modified from Ulrich, 2005)

In the absence of folate, deoxyuridylate (dUMP) levels rise and results in dUMP being used in place of thymidylate (dTMP) during DNA synthesis (Mashiyama et al, 2008). This can ultimately result in DNA damage through mutations and or chromosome breakage (Blount et al, 1997). In addition to accumulation of dUMP, folate deficiency can lead to a failure to convert homocysteine to S-adenosylmethionine. The inability to methylate DNA has a variety of cellular consequences, including the alteration of gene expression, chromosome damage, and increased rates of gene mutation (Zingg and Jones, 1997). Moreover, changes in protein methylation, in particular methylation of Ras, has been detected, which has been associated with altered function (Winter-Vann et al, 2003).

Folate and Cancer

Epidemiological studies have suggested that a diet low in unsaturated fat, rich in fruits and vegetables, and containing adequate amounts of calcium, fiber, and vitamins, including folate can reduce cancer risk (Gridley et al, 1992; Willett et al, 1990; Lee et al, 2006). There have been numerous studies reporting a link between folate status and certain types of cancer including colorectal, breast, cervical, esophageal, gastric, hepatic, lung, pancreatic, neuroblastoma, and leukemias (Lin et al, 2006). In one study, researchers observed a 40% reduction in colorectal cancer risk in individuals that obtained a high amount of folate (Kim, 2004).

Strong evidence for a link between folate deficiency and colorectal cancer was provided in the study by Chen (Chen et al, 1996) in which certain polymorphisms in the gene coding for 5,10-methylenetetrahydrofolate reductase were detected with a high frequency in individuals with colorectal cancer. In another study, knockout mice were created that were heterozygous for the folate transporters Folbp1 and Rfc1 (Ma et al, 2005). This effectively reduced the number of folate transporters in these mice, allowing researchers to investigate the effects of folate deficiency on colon cancer. As a result of this downregulation, the expression of particular genes associated with colon cancer, including *Cdh1*, *Dcn, Esr1, Igf2*, and *Pik3cg*, were altered. An increase in cell proliferation within colonocytes was also detected in the knockout mice when compared to wild type. Upon inspection of colon tissue in the knockout mice, an increase in lesions as well as tumors were detected.

WNT Ligands and Signaling

There are currently 19 known Wnt genes, coding for a like number of signaling proteins. Wnt proteins are secretory and have been shown to be localized many cells away from their sites of origin (Nusse, 2005). These proteins have a variety of roles and functions in cell growth, movement, orientation, and differentiation. Generally, these various functions can each be regulated by any one particular Wnt protein. The Wnt proteins are highly conserved, indicating an essential role in normal cellular growth and maintenance in eukaryotes (Miller et al, 1999).

There are two primary Wnt pathways: the canonical pathway and the non-canonical or calcium pathway. In the canonical pathway (Fig. 2), a Wnt ligand binds to the cell surface receptors frizzled (FRZ) and low density lipoprotein receptor-related protein (LRP). This in turn promotes the formation of the Wnt receptor complex by Axin associating with Disheveled (DSH) and the LRP receptor. In unstimulated cells, Axin, Glycogen Synthase Kinase 3 (GSK-3), and Adenomatosis Polyposis Coli (APC) form a complex that leads to the phosphorylation of β-catenin by GSK-3. β-catenin is then ubiquitinated and degraded by proteolytic activity. The formation of the Wnt receptor complex inhibits the activity of the axin, GSK-3, APC complex. Consequently, cytoplasmic β-catenin levels rise due to reduced degradation. The β-catenin moves into the nucleus where it interacts with T-cell factor (TCF) and binds to specific gene promoters activating transcription of target genes, such as *c-myc*, *cyclin D*, and *c-jun* (Polakis, 2000).

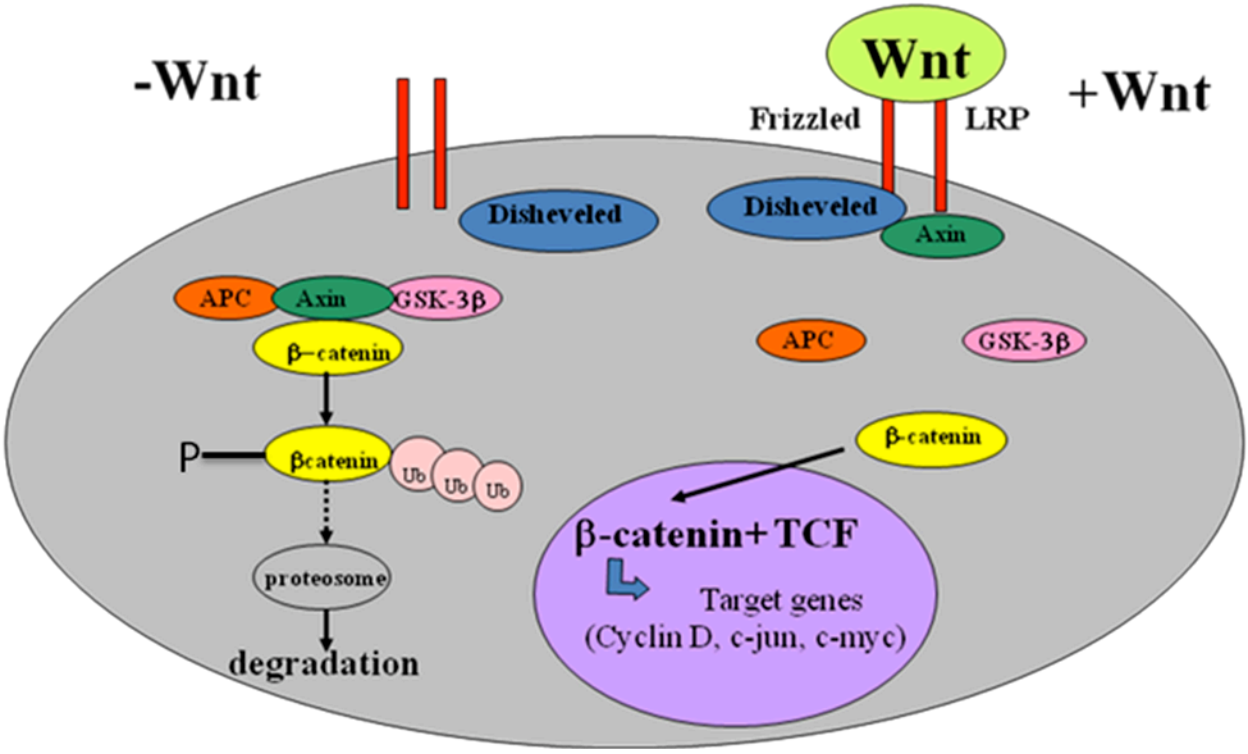


Fig. 2. The Canonical Wnt Signaling Pathway. (Modified from www.stanford.edu/~rnusse/pathways/celldouble.html)

There are many ways in which the non-canonical Wnt pathway can be activated. Each begins with the binding of a Wnt ligand to a cell surface receptor and through a series of events intracellular calcium concentrations rise, Phospholipase C, Protein Kinase C, and the transcription factor NF-AT are activated (Veeman et al, 2003). It has been suggested that some intermediates of the non-canonical pathway are able to inhibit the canonical pathway (Topol et al, 2003).

WNT Signaling and Cancer

Abnormal expression of Wnt proteins has been found to be associated with developmental defects and changes in cell growth and function. Various studies have

shown that an increase or decrease in the expression of certain Wnt genes in various forms of cancer (Moon et al, 2002). In colorectal cancer, APC mutations are nearly always detected in tumor tissue (Luebeck and Moolgavkar, 2002). APC would normally be involved in the ubiquitination and subsequent degradation of β-catenin, thus reducing gene expression. Reduced levels of APC result in increased amounts of β-catenin, ultimately leading to increased expression of Wnt target genes (Shiratsuchi et al, 2007).

Lugli et al. (2007) performed microarray studies of colorectal tumors and observed decreased APC expression, which resulted in increased nuclear β-catenin levels. Tumors with lower amounts of cytoplasmic APC and higher amounts of nuclear β-catenin were associated with increased invasiveness, poorer prognosis, and increased transcription of β-catenin target genes.

To further support the hypothesis that increased nuclear β-catenin results in altered transcription in colorectal cancer, Dillard and Lane (2007) treated a colon cancer cell line with retinol and observed a significant decrease in nuclear β-catenin localization. Researchers then measured the amount of β-catenin induced transcription as well as mRNA levels of genes involved in cell proliferation and found a significant decline in gene expression.

Linking Folate Deficiency, the Wnt Pathway, and Cancer

As discussed, reduced folate levels have profound cellular effects that may include increased DNA damage and mutation rates, in addition to altered methylation patterns due to reduced S-adenosylmethionine. As a secondary effect, changes in gene expression have been detected in folate-deficient cells (Crott et al, 2007; Katula et al,

2007; Zhenhua et al, 2007). It is likely that one or more of these alterations are responsible for the link between folate deficiency and cancer, in particular colorectal cancer, although the causative mechanism is not known. One possibility not thoroughly explored is that changes in specific cell signaling pathways that occur as a response to cells adapting to low folate conditions results in cancer.

A microarray study comparing folate-deficient to folate-sufficient cells revealed that expression of particular Wnt signaling pathway genes were altered (Katula et al, 2007; Crott et al, 2007). Up-regulated genes included both *Wnt5a* and *Wisp1*. *Dkk1*, a Wnt pathway inhibitor, was shown to be down-regulated (Katula et al, 2007). In a more recent study, upregulation of both APC and β-catenin mRNAs were detected as folate levels were decreased in normal human colon cell lines, again showing an association between folate status and the Wnt pathway in cell culture (Crott et al, 2007). In an additional study, depletion of folate and other B vitamins resulted in an increase in neoplastic transformation in the colon cells of mice, this also resulted in global DNA hypomethylation, and alterations to the Wnt pathway, including APC and β-catenin (Zhenhua et al, 2007).

Additional indirect evidence that the folate pathway may be linked to the Wnt pathway is derived from investigation of gene expression in mice that exhibited hyperhomocysteinemia (Ernest et al, 2002). As previously mentioned, 5-methyl-tetrahydrofolate is used to convert homocysteine into methionine. Mice with elevated homocysteine levels produce a phenotype which is very similar to that of mice with certain Wnt pathway abnormalities. Gene expression profiles confirmed that mice with

mutations in the Wnt pathway have higher homocysteine levels. In another study, a mouse genetic model for a folate-responsive neural tube defect, crooked tail (cd) was found to have a mutation in a Wnt coreceptor, LRP6, which eliminates its regulation by the Dkk1 Wnt ligand (Carter et al, 2005). These findings provide further support for a link between the folate metabolic pathway and Wnt signaling.

It is the major aim of this study to determine the effect of folate deficiency on the Wnt signaling pathway.

CHAPTER II

MATERIALS AND METHODS

Preparation of Custom Medium

Delbecco's Modified Eagle's Medium (DMEM) was prepared using the formulation of the American Type Culture Collection (ATCC) (Appendix A) with the exception that folate was initially excluded. After mixing, the medium was equally separated into two batches. Folate was added to one batch (2 mg/L) but not the other. The medium was filter sterilized (0.20 μm filter). Complete medium was made by adding 50 ml of filter sterilized dialyzed calf serum (dcs) to 450 ml of DMEM from each batch, along with 5 ml of 100X Penicillin/Streptomycin (10,000 IU/ml 10,000 μg/ml). This medium is referred to as DMEM Complete With (folate) and DMEM Complete No (folate).

Cell Lines

NIH 3T3 cells transfected with the TOP/FOP Flash reporter vectors (obtained from Randall Moon, University of Washington, Howard Hughes Medical Institute, Department of Pharmacology) were used as a cell model. The cell lines are referred to as TOPFlash-3T3 and FOPFlash-3T3. The TOP/FOP Flash reporter system allows for the determination of β-catenin activity. The TOPFlash construct has three β-catenin binding sites in the promoter of the coding region of a luciferase gene. Increased binding of β-catenin results in the increased expression of luciferase. The FOPFlash reporter construct serves as a control for the TOPFlash plasmid. The β-catenin binding sites in the

FOPFlash plasmid have been mutated so that β-catenin will not bind, and essentially no luciferase should be transcribed.

Cell Culture

TOPFlash 3T3 and FOPFlash 3T3 cells were grown in DMEM Complete medium until 80% confluence. On day 0, the cells were collected by trypsinizing and replated. Cells were plated at a starting density of 5.6 X 10^5 cells per T-75 flask and brought up to 12 ml with either DMEM Complete, with or no. 10 μl of G418 was then added to each T-75 flask. Cells were grown at 37° C in a 5% carbon dioxide atmosphere.

On day 2 of the culture period, the cells that were utilized for the second time point (day 8) were collected and re-plated at 5.6 X 10^5 cells per flask. On the same day, cells to be used for the first time point (day 4) were collected and re-plated for treatment with conditioned medium. The following day (day 3), the cells for the first time point were treated with either L-cell or Wnt3a conditioned medium (see preparation of conditioned medium), which was diluted 1:1 with fresh medium (DMEM Complete No). The medium was removed from cells and replaced with conditioned medium. Cells for the first time point were then collected on day 4. Also on day 4, cells used for the second time point (day 10) were collected and re-plated at 5.6 X 10^5 cells per flask. At day 6, the cells used for the day 8 time point were again collected and re-plated for treatment with conditioned medium. The following day (day 7), the cells were treated with either L-cell or Wnt3a conditioned medium, which was diluted 1:1 with fresh medium (DMEM Complete No). The medium was removed from cells and replaced with conditioned medium. Cells were collected at day 8. Cells for the second time point (day 10) were re-

plated for treatment on day 8. The following day (day 9), the cells were treated with conditioned medium. Cells were then collected on day 10. Collected cells were assayed for luciferase, Western blot, or immunofluorescent analysis.

Preparation of Conditioned Medium

Two cell lines were obtained from the American Type Culture Collection (ATCC), L-cell with a Wnt3a expression vector (CRL-2647) and a normal L cell line (CRL-2648). Cells were initially grown to near confluency in T-75 flasks. The cells were collected, brought to 10 ml in DMEM Complete, and replated at a 1:10 dilution in ten 100 mm plates. On day 4, the medium was removed, filtered, and stored at 4° C. An additional 10 ml of DMEM Complete was added to each 100 mm dish and the cells were allowed to grow for an additional 3 days. At day 7, the medium was removed, filtered, and combined with the medium collected on day 4. The medium was then stored at -20° C.

In Situ Immunofluoresence

TOPFlash 3T3 cells were grown for 4, 8, and 10 days in folate-deficient and sufficient medium. Two days prior to collection, the cells were grown directly on coverslips in 24 well dishes. The day prior to collection, the cells were treated with Wnt3a or L-cell conditioned medium as previously described. Cells were fixed directly in 24-well plates with 1 ml 2% formaldehyde in phosphate buffered saline (PBS) for 15 minutes. Following fixing, cells were washed three times with 500 μl PBS for five minutes per wash. Cells were stored in 500 μl PBS at 4°C.

Cells were permeabilized with 1 ml 0.25% Triton X-100 diluted in PBS for 5

minutes. Following permeabilization, cells were washed three times with 500 μl PBS. 50 μl of a 20 μg/ml dilution of a mouse anti-β-catenin antibody (Zymed Laboratories, catalogue# 138400) was added directly onto the cells and incubated at 37° C for 90 minutes. Following incubation, cells were washed three times with PBS for 5 minutes per wash. 50 μl of a secondary antibody, goat anti-mouse IgG, with fluoroform Alexa 488 (Invitrogen, catalogue# A11001), diluted 5 μg/ml was then applied. Cells were then shielded from light and incubated at room temperature for one hour. Cells were then washed three times with 500 μl PBS for five minutes per wash. Nuclei counterstaining was performed with 300 μl of a 1:100,000 dilution of DAPI for 5 minutes at room temperature. Cells were then washed twice with 500 μl PBS for 5 minutes. Coverslips were mounted on glass slides using Dako Cytomation Fluorescent Mounting Medium (catalogue# S3023). Cells were then imaged using confocal microscopy (Olympus FV500) and the Fluoview imaging software (Olympus). A single plane of .5 μm was used for imaging. Quantification of imunofluorescent signal was obtained using the ImageJ software. Average nuclear signal was measured along with average cell signal.

Luciferase Assay

Cells used for luciferase assay analysis were collected by trypsinization, resuspended in PBS, and counted. Equal cell numbers were aliquoted into glass tubes and the cells pelleted by centrifugation at 2,000 rpm. The supernatant was removed and the cells resuspended in 1X Cell Lysis Buffer (Promega, Inc) for 15 minutes at 37° C . Lysates were transferred to microcentrifuge tubes and stored at -20° C. Luciferase was measured using the Luciferase Assay System (Promega, Inc.). 20 μl of cell lysate was

pipeted into luminometer tubes. Readings were then taken using a Berthold LB 9501 luminometer. Variability in cell number between samples was accounted for by measuring DNA and standardizing luciferase results to quantified DNA (see DNA quantification).

Western Blot

TOPFlash 3T3 cells were grown for 4, 8, and 10 days in folate-deficient and sufficient medium. Two days prior to collection cells were grown in 100 mm dishes. The day prior to collection, each group of cells was stimulated with either a Wnt3A conditioned medium, or an L-cell control.

Cells used for Western blot analysis were collected by trypsinization, resuspended in PBS, and counted. Equal cell number was aliquoted into glass tubes tubes and the cells pelleted by centrifugation. The supernatant was removed and the cells resuspended in Cell Lytic^{TM} M Cell Lysis Reagent (Sigma, catalogue# C2978). Samples were incubated at room temperature for 15 minutes and transferred to microcentrifuge tubes. Lysates were centrifuged at 14K for 5 minutes, the supernatant transferred to a clean microcentrifuge tube and stored at -80°C. Protein content of the samples was then determined (see protein quantification).

An equal amount (equal cell number or equal amount of cellular protein) of each sample group was loaded into separate wells of an acrylamide gel (10%). Prestained molecular weight markers (Colorburst; Sigma, catalogue# C4105) was loaded into one lane of the gel. Electrophoresis was performed for 3 hours at 21 mA.

Following electrophoresis, protein was transferred to nitrocellulose using standard

methods.

The next day, the blot was initially stained in Ponceau S (1% in 1% acetic acid diluted 1:10 with ddH_20) to visualize the proteins and confirm equal loading. The blot was incubated in 5% milk in Tween-Tris Buffered Saline (TTBS) (.15M NaCl, .01M Tris pH 8.0, 500 μl Tween) for one hour. A 1:500 dilution of mouse anti-β-catenin (Zymed Laboratories, catalogue# 138400) and 1:200 dilution of mouse anti-tubulin antibodies (Developmental Studies Hybridoma Bank) in 1% milk in TTBS was added to the blot for one hour. A 1:500 dilution of mouse anti-LRP6 (Santa Cruz Biotechbnology Inc, catalogue# sc-25317) and a 1:200 dilution of mouse anti-tubulin antibodies in 1% milk in TTBS was added to the blot for one hour to determine LRP6 content. The nitrocellulose filter was then washed in TTBS three times for five minutes and once for 15 minutes. A 1:10,000 dilution of goat anti-mouse horse radish peroxidase (Sigma, catalogue# A3673) in 1% milk in TTBS was placed on the nitrocellulose for one hour. The nitrocellulose was again washed three times in TTBS for 5 minutes and once for 15 minutes. The Supersignal West Femto Chemiluminescent detection kit (Pierce, catalogue# 34095) was used to detect the antibody on the nitrocellulose. Imaging was performed on a Biorad Chemi Doc XRS imager using the Quantity One software. Variation in loading was accounted for by quantifying tubulin and standardizing β-catenin and LRP6 accordingly.

Protein Quantification

Protein content of the Western blot samples were determined as follows: Generally, 10 μl of cell lysates were added to 790 μl of ddH2O and 200 μl of Bradford dye (Bio Rad, catalogue# 500-0006) was added. A bovine serum albumin (BSA) standard

of 0, 2, 5, 10, 15, and 20 μg of protein was also prepared. Light absorbance was measured at 595 nm using a Genesis 10UV spectrophotometer. Unknown protein contents were determined using the generated BSA standard curve.

Quantification of DNA

10 μl of each cell lysate sample was placed in a 96 well plate. 50 μl of a DNA binding dye (Invitrogen; CyQuant NF Cell Proliferation Assay Kit, catalogue# C35006) diluted 1:750 in 1X Hank's Buffered Salt Solution (HBSS) was then added to each well. The samples were shielded from light and incubated at 37°C for 15 minutes. Samples were measured on a BIO-TEK Synergy HT microplate reader, excitation set at 485 nm and emission detection at 530 nm using the KC4 software.

Statistics

For the luciferase assay there were 2 biological replicas performed, included in this were 6 technical replicas (for unstimulated cells at day 10 at least 3 technical replicas were performed). *P*-values were determined using a two-tailed student T-test with significance given to values <0.05.

For the Western blot, three biological replicas were used to determine β–catenin protein content. *P*-values were determined using a two-tailed student T-test with significance given to values <0.05. Two biological replicas were used to determine LRP6 protein content and averages were reported.

For immunofluorescent quantification of β–catenin localization, a minimum of 34 cells were analyzed over 2 biological replicas. *P*-values were determined using a two-tailed student T-test with significance given to values <0.05.

CHAPTER III

RESULTS

β–catenin Activity in Folate-Deficient and Sufficient Cells

Based on the finding that Wnt signaling components are altered due to folate deficiency (Katula et al, 2007; Crott et al, 2007; Liu et al, 2007) we decided to determine the effect of folate deficiency on β–catenin activity. It was expected that β–catenin activity would increase.

In order to explore this possibility, NIH 3T3 cells that had been stably transfected with the TOP/FOP Flash reporter system (Katula lab) were used as an experimental system. The TOPFlash plasmid contains a luciferase gene controlled by a promoter with three β–catenin binding sites. Thus, a higher level of luciferase activity indicated more activated β–catenin. The FOPFlash plasmid contains mutated β–catenin binding sites such that β–catenin will not bind and no luciferase will be transcribed, and served as a control.

The TOPFlash-3T3 and FOPFlash-3T3 cells were grown for 4, 8, and 10 days in folate-deficient and sufficient medium using a protocol that maintains subconfluent cell densities (see Materials and Methods). At each time point cells were collected and assayed for luciferase activity. As folate-deficient cells proliferate at a slower rate, particularly after seven days, we controlled for cell number in two ways. First, folate-sufficient and deficient cells were collected, counted, and equal cell number aliquoted.

Second, the DNA content of the cell lysates was determined.

At day 4 of the culture period there was no significant change in β–catenin activity (reported as relative light units per unit DNA) in cells grown under folate-deficient conditions, compared to those grown under folate-sufficient conditions for both Wnt stimulated ($p<0.069$) (Fig. 3) and unstimulated samples ($p<0.481$) (Fig. 4). This lack of differential activity between the folate-deficient and sufficient groups could be due to the fact that with just four days of growth in folate-deficient medium, cellular levels of folate or folate pathway intermediates were not yet exhausted.

At day 8 of the culture period there was a significant change in β–catenin activity when comparing cells grown under folate-deficient and sufficient conditions. There was nearly a 3 fold increase in β–catenin activity in Wnt stimulated folate-deficient cells compared to Wnt stimulated folate-sufficient cells ($p<0.0003$) (Fig. 3). When comparing unstimulated cultures there was no significant difference in β–catenin activity ($p<0.074$) (Fig. 4).

At day 10 of the culture period there was a significant change in β–catenin activity in both Wnt stimulated cultures ($p<0.0003$) (Fig. 3). An unexpected result was obtained in unstimulated cultures, where folate-sufficient cells showed greater β–catenin activity than did folate-deficient cells ($p<0.0281$) (Fig. 4). This could be explained by the low level of luciferase activity due to the cells not being stimulated. These results do show that folate status has an effect on β–catenin activity.

The FOPFlash cells showed little luciferase activity in both stimulated and unstimulated cultures, as expected (data not shown). This result confirms that luciferase

activity observed in the TOPFlash cells was due to increased β–catenin activity in the promoter of the luciferase gene in the TOPFlash plasmid.

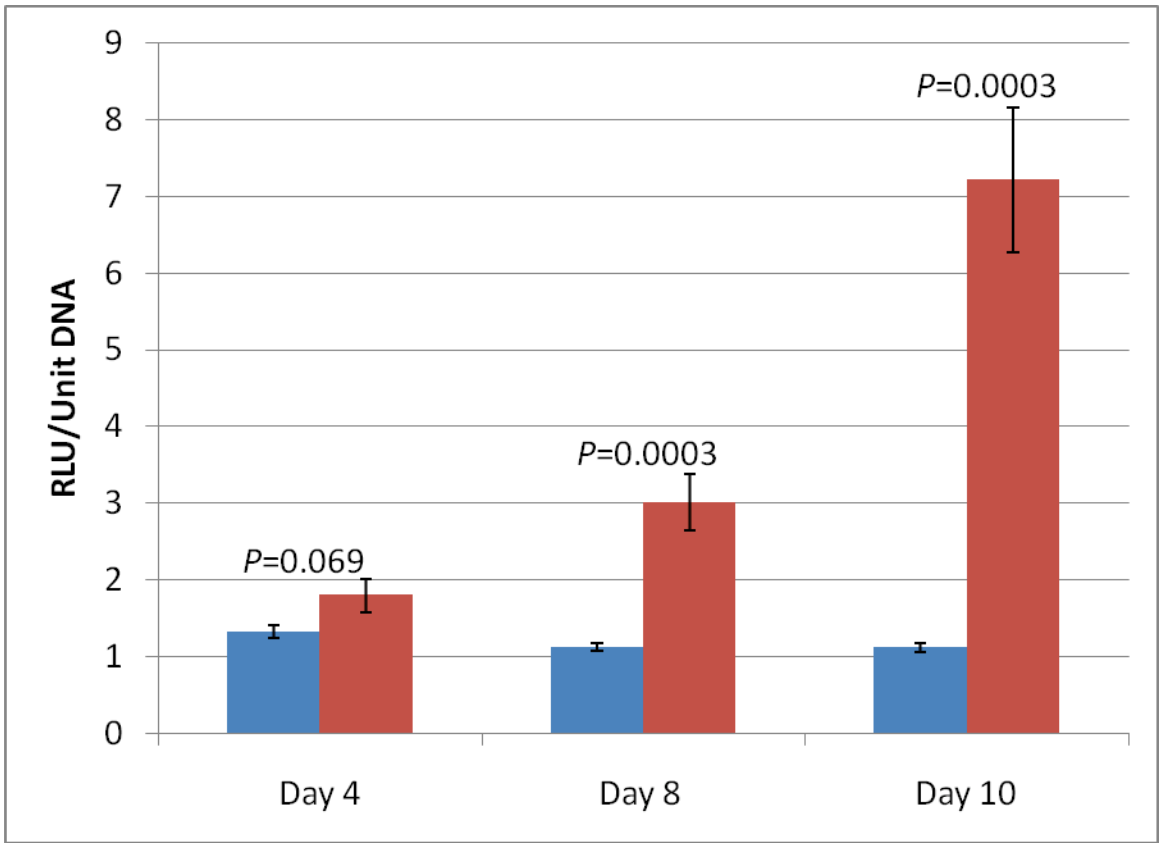


Fig. 3. Alterations in β–catenin Activity in Stimulated Folate-Deficient Cells. β–catenin activity reported as RLU per unit DNA in TOPFlash-3T3 cells stimulated with Wnt3a conditioned medium and grown under folate-deficient (blue) and sufficient (red) conditions for 4, 8, and 10 days. Error bars represent standard error with *P* values reported (n=12).

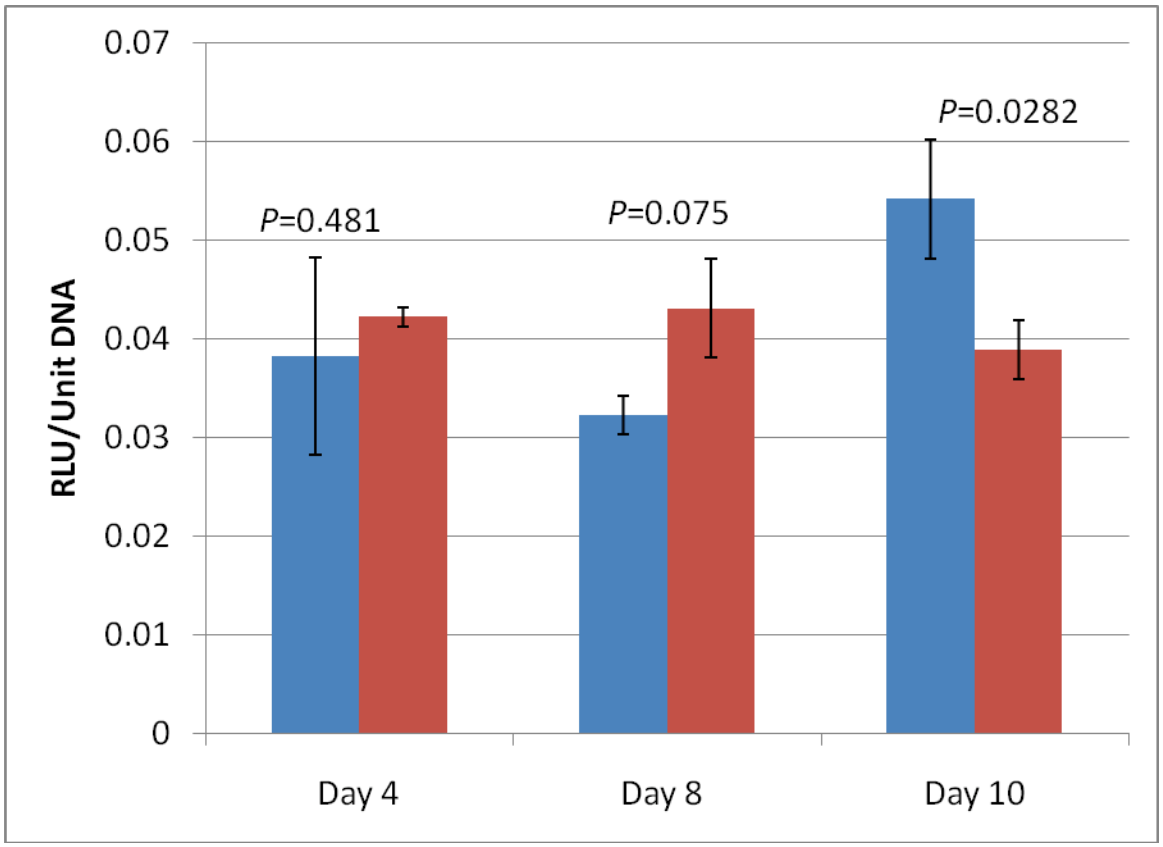


Fig. 4. Alterations in β–catenin Activity in Unstimulated Folate-Deficient Cells. β–catenin activity reported as RLU per unit DNA in TOPFlash-3T3 cells without stimulation and grown under folate-deficient (blue) and sufficient (red) conditions for 4, 8, and 10 days. Error bars represent standard error with *P* values reported (n=12).

β–catenin and LRP6 Protein Levels

One possible explanation for the increase in β–catenin activity in folate-deficient cells is an increase in Wnt pathway receptors; this would allow for more binding of Wnt ligands and result in less β–catenin degradation by proteosomes. Another explanation is an increase in the amount of β–catenin present in folate-deficient cells.

In order to explore these two possibilities, cells were grown under folate-sufficient and deficient conditions for 4, 8, and 10 days. Cells were lysed and a Western

blot performed to determine overall β–catenin and LRP6 protein levels.

Western blot analysis (Fig. 5) of folate-deficient compared to folate-sufficient cells revealed a slight increase in β–catenin protein levels at day 10 when standardized to tubulin, however this increase was not significant. There was no significant or observable difference in β–catenin protein at the day 4 or 8 time periods (Fig. 6).

Fig. 5. Western Blot of β–catenin, LRP6, and Tubulin. Detection of β-catenin and LRP6 protein in Wnt3a stimulated and unstimulated folate-deficient and sufficient NIH3T3 cells. Cells were grown for 4, 8, and 10 days in folate-deficient or sufficient medium as descibed (Materials and Methods) and treated with Wnt3a or L-cell conditioned medium. Cell lysates were prepared and 10μg electrophoresed. TNL; TOPFlash, No folate, L-cell conditioned medium. TWL; TOPFlash, With folate, L-cell conditioned medium. TNW; TOPFlash, No folate, Wnt3a conditioned medium. TWW; TOPFlash, With folate, Wnt3a conditioned medium. The numbers indicate the day of cell collection.

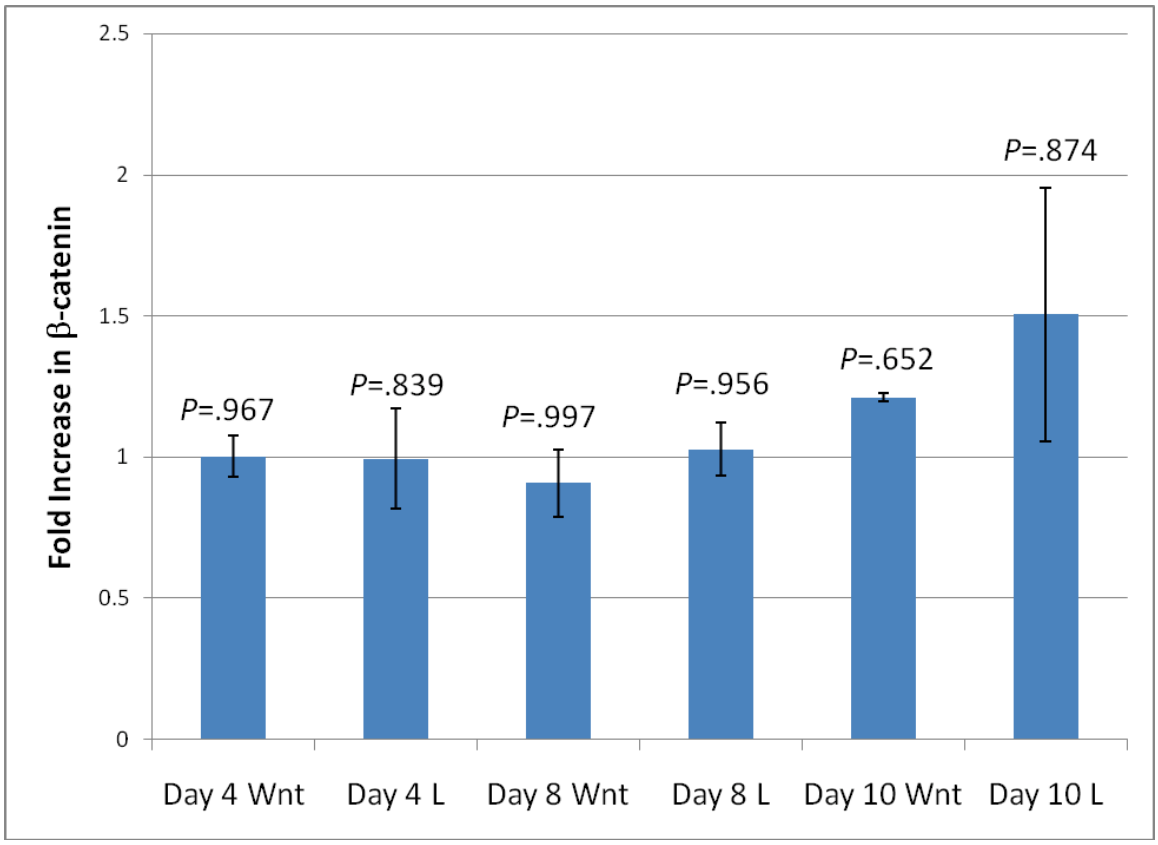


Fig. 6. β–catenin Fold Increase in Folate-Deficient/Folate-Sufficient Cells. Fold increase in β–catenin in folate-deficient cells compared with folate-sufficient cells grown for 4, 8, and 10 days with Wnt3a stimulation (Wnt) or no stimulation (L). Error bars represent standard error with *P* values reported (n=3).

Western blot analysis (Fig. 7) of folate-deficient compared to folate-sufficient cells revealed no signficant difference in LRP6 levels at the day 4, 8, or 10 time periods.

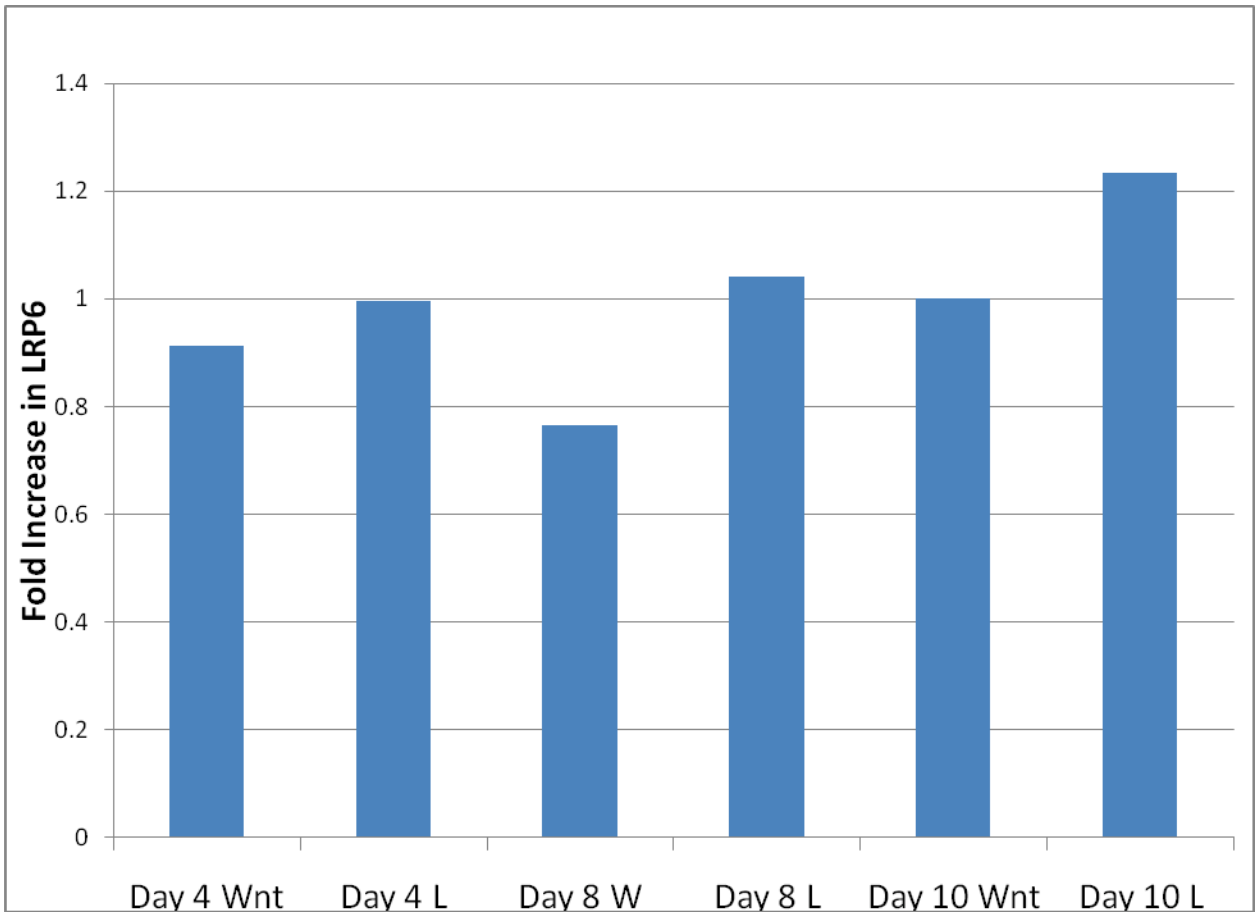


Fig. 7. LRP6 Fold Increase in Folate-Deficient/Folate-Sufficient Cells. Fold increase in LRP6 in folate-deficient cells compared with folate-sufficient cells grown for 4, 8, and 10 days with with Wnt3a stimulation (Wnt) or no stimulation (L). Results are reported as an average (n=2).

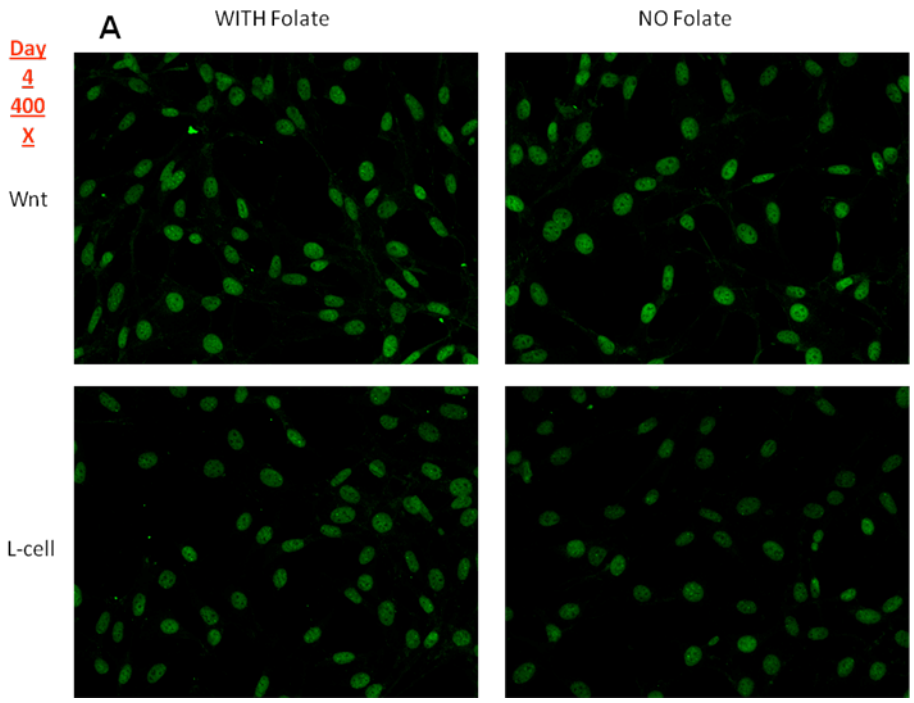
β–catenin Localization

We found that β–catenin activity increases in folate-deficient cells but there was no significant change in Wnt pathway receptor LRP6 and β–catenin protein levels. One explanation is that a greater fraction of β–catenin is localizing to the nucleus. β–catenin cellular localization was examined by immunofluorescence.

Cells were grown under folate-sufficient and deficient conditions for a period of

4, 8, and 10 days. Cells were fixed, immunostained for β–catenin, and analyzed by confocal microscopy (Fig. 8). Total cell signal and nuclear signal were quantified and the proportion of nuclear/cell signal determined as a measure of β–catenin protein distribution.

Analysis of immunofluorescent images at all three time points (day 4, 8, and 10) showed an increase in nuclear localization of β–catenin in Wnt stimulated cells when compared to unstimulated cells regardless of folate status. This is as would be expected, since activation of the Wnt pathway by Wnt3a ligand binding leads to stabilized β–catenin and increased nuclear localization.



(Fig. 8 legend next page)

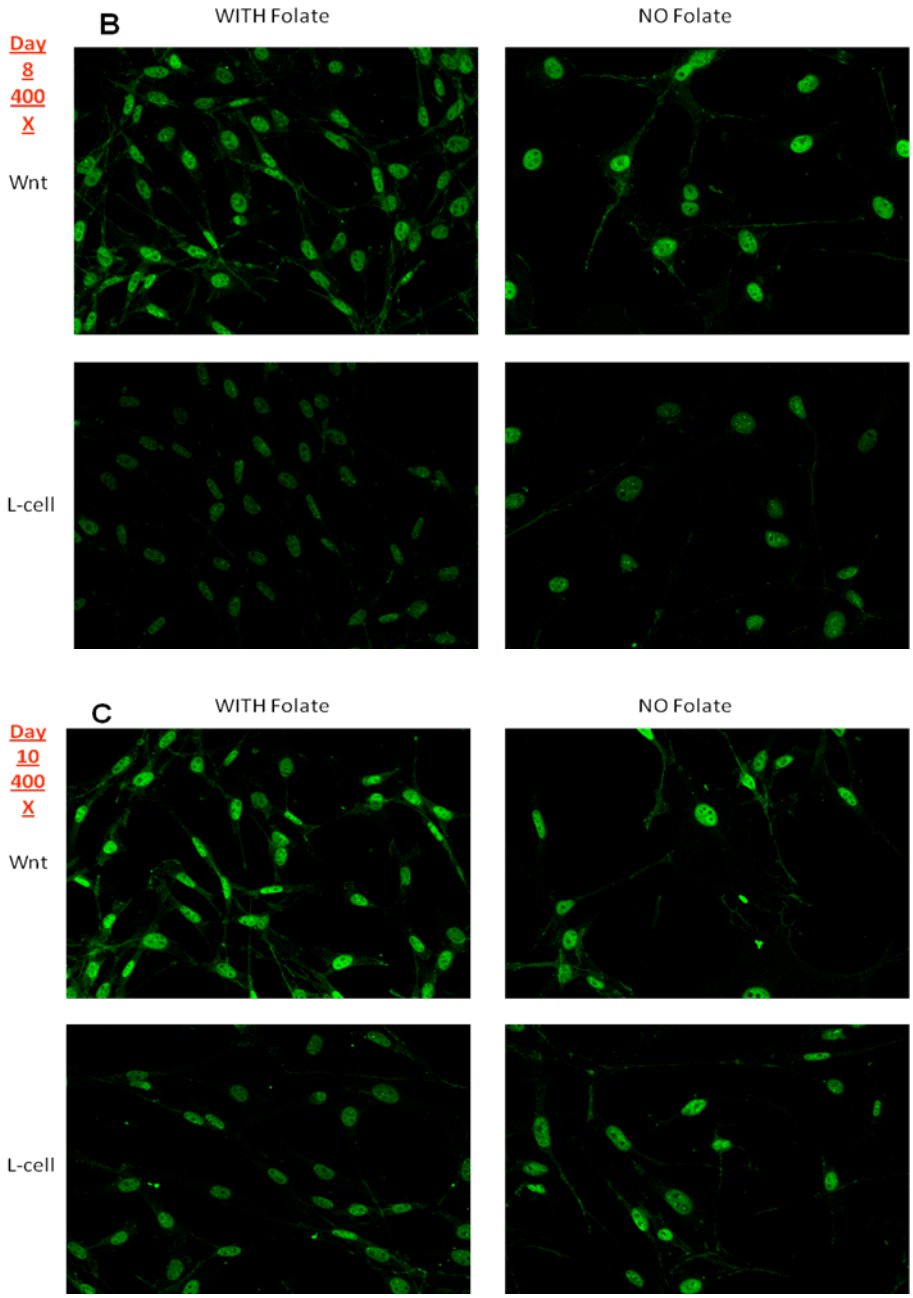


Fig. 8. Immunofluoresent Staining for β–catenin Localization. A) immunofluorescent staining of cells grown for 4 days under folate-sufficient (with) or deficient (no) conditions and either stimulated (Wnt3a) or unstimulated (L-cell). B) cells grown for 8 days under with/no and Wnt/L-cell conditions. C) cells grown for 10 days under with/no and Wnt/L-cell condition. Cells were immunostained for β–catenin.

When quantifying nuclear signal to overall cell signal per unit area for day 4 samples there was an increase in β−catenin nuclear localization in folate-deficient cells compared to folate-sufficient cells (Fig. 9). However, the reported increase in nuclear localization is not significant in unstimulated cells (p<0.137). In Wnt stimulated cells, a significant difference in nuclear localization was observed (p<0.026).

Quantitative analysis of day 8 samples show a significant increase in nuclear β−catenin localization in folate-deficient Wnt3a stimulated cells versus stimulated folate-sufficient cells (p<0.000003). Unstimulated treatment groups did not show a significant increase in nuclear localization (p<0.069) (Fig. 9). Although not significant, there was still an observed increase in nuclear localization of β−catenin in unstimulated folate-deficient cells compared with unstimulated folate-sufficient cells.

Quantitation of day 10 samples show a significant increase in nuclear β−catenin localization in both stimulated (p<0.00003) and unstimulated cells (p<0.002) when comparing folate-deficient cells to folate-sufficient cells (Fig. 9).

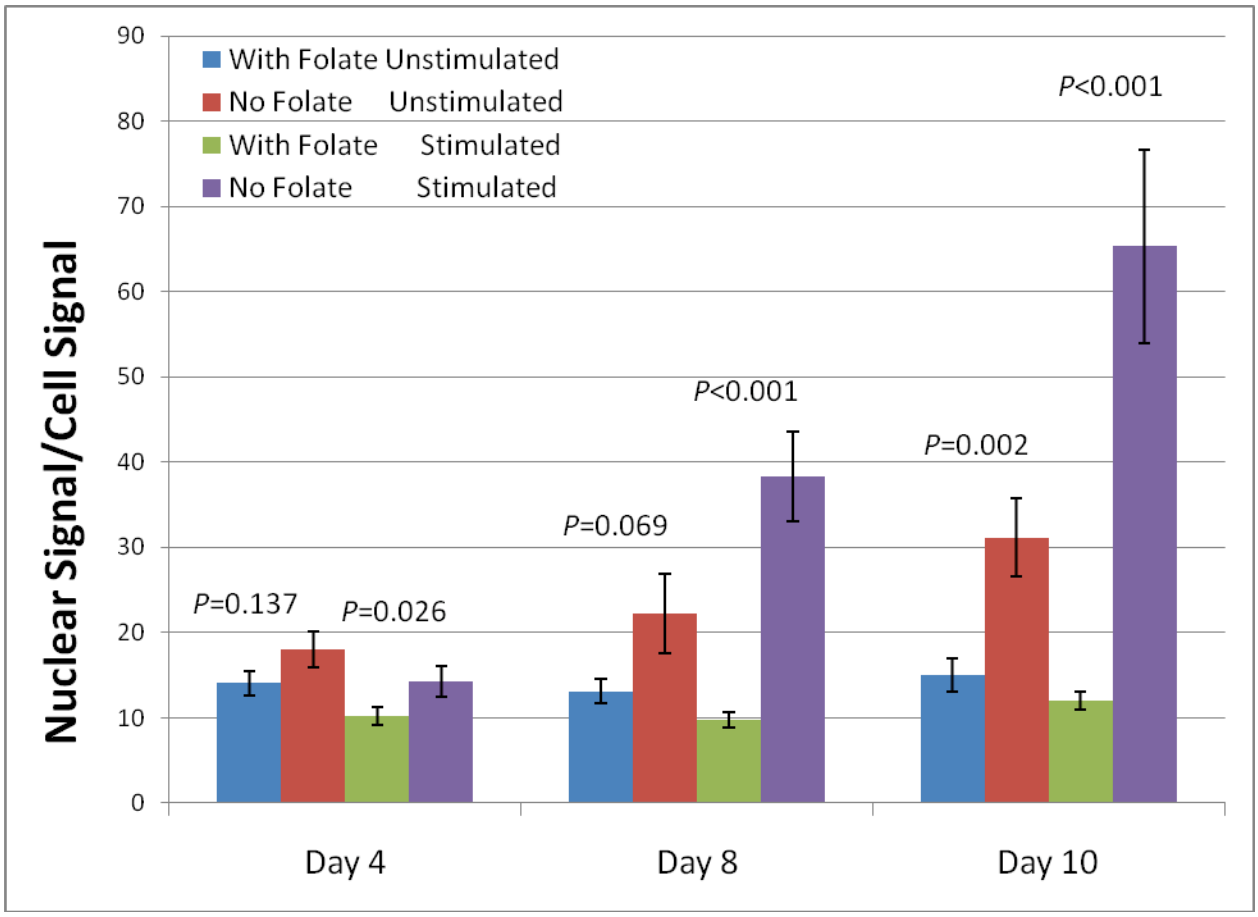


Fig. 9. Nuclear Localization/Cytoplasmic Localization of β–catenin. NIH3T3 cells grown for 4, 8, and 10 days under folate-sufficient and deficient conditions. Nuclear localization of β–catenin reported as nuclear signal per unit area/cytoplasmic signal per unit area. Error bars represent standard error with *P* values reported (n>80).

CHAPTER IV

DISCUSSION

It has been reported that folate deficiency leads to an increased risk for various cancers (Lin et al, 2006). The strongest association is that between folate status and colorectal cancer (Kim, 2004). It has also been observed that certain Wnt pathway abnormalities are present in many cancers, including colorectal cancer (Lugli et al, 2007). Microarray studies of folate-deficient cells have suggested a strong association between folate status and alterations in the Wnt signalling pathway (Crott et al, 2007; Katula et al, 2007). In vivo studies of folate deficiency have also shown alterations in the Wnt signaling pathway (Zhenhua et al, 2007). The mechanism for this association has never been fully explored. The data collected in this study shows a distinct association between between folate status and alterations in the canonical Wnt signalling pathway through changes in β−catenin activity and localization.

A significant increase in β−catenin transcriptional activity was observed within eight days of growth under folate-deficient conditions. This increase in activity shows a correlation between folate status and the canonical Wnt signalling pathway. The increase in β−catenin activity can be explained in many different ways. The possibilities examined in this study were: 1) an increase in the amount of β−catenin protein; 2) an increase in the Wnt signalling receptors; 3) an increase in β−catenin nuclear localization.

An increase in the β−catenin protein was observed in folate-deficient cells

compared to folate-sufficient cells, however, the difference was not significant. Likewise, there was no significant change in the amount of LRP6 receptor proteins in folate-deficient cells compared to folate-sufficient cells. This result shows that the observed increase in β−catenin transcriptional activity was not due to an increase in these Wnt pathway proteins.

Since β−catenin is a transcription factor, it must localize to the nucleus to have a transcriptional effect. It was observed that there was a significant difference in nuclear localization of β−catenin as early as day 4 of growing Wnt stimulated cells under folate-deficient conditions. Active β−catenin would normally be located in the nucleus associating with TCF to promote transcription (Polakis, 2000). In unstimulated cells, β−catenin is found in the cytoplasm, associated with the axin, GSK-3, APC complex, which leads to it's ubiquitination and degradation via proteosomes. β−catenin can also be found associated with the cell membrane and E-cadherin (Lugli et al, 2007). The increased localization of β−catenin to the nucleus and the lack of an increase in the β−catenin protein indicates a reduction in cytoplasmic and cell membrane associated β−catenin. Although the mechanism directing β−catenin nuclear localization is not completely understood, it does appear the folate status may play a role in enhancing or directing the effect. It was shown in hepatocarcinomas that the level of E-cadherin decreases and increased localization of β−catenin to the nucleus occurs (Wilhelm et al, 2003). Insulin-like growth factor 1 (IGF-1) has been shown to play a role in the localization and activity of β−catenin through causing an association of β−catenin and E-cadherin at the cell membrane (Playford et al, 2000). It has also been shown that folate

status plays a direct role in IGF-1 protein expression (Istiadjid, 2005). It is plausible that folate deficiency may cause a decrease in the IGF-1 protein which ultimately could result in β−catenin dissociating from E-cadherin and the cell membrane and localizing to the nucleus. This hypothesis could be further examined by measuring the level of IGF-1 expression in folate-deficient cells.

The other Wnt pathway components that should be studied, includes the FRZ receptor. An increase in the amount of FRZ receptor proteins could explain the increase in β−catenin activity via increased nuclear localization. In fact, in a study by Bliek et al (2004), the FRZ (Frizzled) protein was upregulated during embryogenesis under folate-deficient conditions.

Another Wnt pathway component that could effect β−catenin activity and not cause an increase in the β−catenin protein would be the cofactor T-cell factor (TCF). An increase in the nuclear amount of TCF could result in increased transcription of Wnt pathway target genes, independent of β−catenin protein levels. APC levels have been suggested to be altered in colorectal cancer (Lugli et al, 2007). APC is involved in the complex which degrades β−catenin. An increase in APC would result in β−catenin degradation, however a decrease in APC would result in the stabilization of β−catenin. In addition, APC, has been shown to bind to the enhancer of the *c-myc* gene (Sierra et al, 2006), a gene that is overexpressed in a wide range of cancers and which β−catenin plays a role as a transcription factor. The GSK-3β protein could also effect β−catenin activity and localization independent of the amount of β−catenin protein present. GSK-3β is another protein involved in the complex which results in β−catenin degradation. GSK-3β

causes β−catenin to be degraded through phosphorylation which in turn results in β−catenin being ubiquitinized and subsequently broken down by proteosomes. A loss of GSK-3β activity would result in β−catenin stabilization.

Another possiblility in how folate deficiency may effect the Wnt signaling pathway is through global hypomethylation. Due to reduced intracellular folate levels, a reduction of S-adenosylmethionine (SAM) would result. As previously discussed, SAM is the major methyl donor for all methylation reactions. Global hypomethylation has been associated with improper gene regulation and cancer. Hypomethylation has also been positively associated with the ability of TCF to bind to the promotors of Wnt target genes (Wohrle et al, 2007). A lack of methylation would result in more TCF binding, in the presence of additional β−catenin, this would result in increased transcription of Wnt target genes.

In a study by Zhenhua et al (2007) folate depletion in mice did not result in significant global hypomethylation, however, when combined with the reduction of other 1-carbon nutrients such as riboflavin, viatmin B-6, and vitamin B-12, a nearly 50% reduction in genomic DNA methylation was observed. It was observed that these 1-carbon dietary depletions resulted in increased proliferation of colonic cells. In addition, there were notable changes in the Wnt signaling pathway. One change in the Wnt signaling pathway that was observed was in increase in β-catenin when mutiple 1-carbon compounds were depleted. An increase in β-catenin nuclear localization was also observed. These *in vivo* studies confirm the findings of this study, that folate status has an effect on the Wnt signaling pathway.

For future studies, levels of other Wnt signaling proteins, including: FRZ, APC, GSK-3β, and TCF will be analyzed. In addition, proteins normally seen to associate with β–catenin and control its localization could also be analyzed, these include E-cadherin and IGF-1.

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APPENDIX A. DMEM CUSTOM MEDIUM

The following ingredients are combined in water to a volume of 4 liters. The pH of the medium is then adjusted to between 7.2 and 7.6 using 1N HCl. Medium is then filter sterilized using a .2 um filter.

Inorganic Salts	mg
CaCl2 2H2O	1059.68
Fe(No3)3 9H2O	0.4
KCl	1600
MgSO4 7H20 (dried)	308.04
NaCl	25,600
NaH2PO4 H2O	500
Amino Acids	
L-Arginine HCL	336
L-Cystine	192
L-Glutamine	2336
Glycine	120
L-Histidine HCl H2O	168
L-Isoleucine	420
L-Leucine	420
L-Lysine HCl	584
L-Methionine	120
L-Phenylalanine	264
L-Serine	168
L-Threonine	380
L-Tryptophan	64
L-Tyrosine	288
L-Valine	376

Vitamins	mg
Choline Chloride	16
Folic Acid	8
myo-Inositol	28.8
Nicotinamide	16
Pantothenate Calcium	16
Pyridoxal HCl	16
Riboflavin	1.6
Thiamine HCl	16
Other components	
D-Glucose	18000
Phenol Red	60
Sodium Pyruvate	440
Sodium Bicarbonate	14800
Hepes	14280

http://www.atcc.org/common/documents/pdf/30-2002.pdf